

Decreasing *Streptococcus pyogenes* intracellular infections in RAW 264.7 cells with ML141 and rifampin co-treatment

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ABSTRACT

RESEARCH PAPER: Decreasing *Streptococcus pyogenes* intracellular infections in RAW 264.7 cells with ML141 and rifampin co-treatment

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Streptococcus pyogenes infections cause over 517,000 deaths per year. Treatments typically fail to eradicate *S. pyogenes* bacteria, resulting in recurrent infections. Therefore, we are investigating the effects of rifampin and ML141 co-treatment on *S. pyogenes* intracellular infections and toxicity of these treatments in RAW 264.7 cells. Rifampin is an antibiotic, whereas ML141 is a molecule that inhibits *S. pyogenes* invasion. Our experiments will be conducted by infecting RAW 264.7 cells with *S. pyogenes* bacteria and treating them with rifampin alone, ML141 alone, or rifampin with ML141 co-treatment. Also, cytotoxicity assays will be performed on rifampin and ML141 co-treatment, rifampin alone, and ML141 alone, using a flow cytometer. It is our hypothesis that rifampin and ML141 co-treatment will have the lowest *S. pyogenes* bacterial count. We also hypothesize rifampin and ML141 co-treatment will not be toxic. If both hypothesis are supported by the data *S. pyogenes* recurrent intracellular infections will be decreased. Furthermore, rifampin and ML141 co-treatment could be used as a better therapeutic approach for *S. pyogenes* recurrent infections.

Introduction

Streptococcus pyogenes (Group A) is a bacterium that can cause a wide variety of diseases in humans of all ages; however, children under the age of 15 are most likely to be infected [1]. Unfortunately, there is a lack of treatment for patients with recurrent *S. pyogenes* infections, resulting in failure rates as high as 100% [2]. *S. pyogenes* can form biofilms, which could be the explanation for treatment failures and reoccurrences of these bacterial infections [3]. Group A *Streptococcus* also avoids antibiotic treatments by hiding intracellularly in phagocytic cells [4]. Both hypotheses could be the reason for recurrent *S. pyogenes* infections. Although our approach does not target biofilms, it does focus on intracellular infection. Our lab believes that by targeting *S. pyogenes* intracellular infections therapeutically, reoccurring infections could be prevented.

ML141 helps limit the intracellular infection of *S. pyogenes* bacteria, but does not get rid of all the bacteria within the host cell (unpublished). Rifampin is a bactericidal antibiotic and works well in treating intracellular infection but is also cytotoxic when used in high concentrations [5]. Therefore, it is proposed that rifampin and ML141 used as a co-treatment could decrease *S. pyogenes* bacteria. We hypothesize that the amount of rifampin needed to clear infection of *S. pyogenes* within the host cell could be decreased with the co-treatment of ML141, and therefore decrease toxicity. Rifampin and ML141 co-treatment could potentially stop recurrent infections.

The focus of this thesis proposal is to examine this hypothesis. The experimental design is to count colonies on blood agar plates to detect what concentration of rifampin and ML141 co-treatment results in the greatest decrease in *S. pyogenes* intracellular bacteria.

Materials & Methods

Chemicals. In the investigation on inhibiting *S.pyogenes* intracellular infections, the following were used at the appropriate concentration and duration provided in figure1 and methods below:

THB- Todd Hewitt Broth, dimethyl sulfoxide- DMSO, sterile water, agar plates, 0.85% saline (Thermo-Fisher Scientific Waltham, MA), RAW 264.7 cells (American Type Culture Collection, ATCC, Manassas, VA), rifampin (MP Biomedicals, Santa Ana, CA), ML141 (Dr. Sammelson, Department of Chemistry, Ball State University), rifampin and ML141 co-treatment, gentamicin (Sigma Aldrich, St. Louis, MO), and sheep-blood (Cleveland Scientific, Cleveland, OH).

RAW Cell Culture. *S. pyogenes* were cultured (200 rpm, 37°C, 24 hr) in Todd Hewitt Broth (Fisher, #DF0492-17-6) and subcultured before doing the assay.

Invasion Assay. To begin the assay, bacteria were spun (10000 rpm, 37°C, 3 min) and washed. The appropriate concentration of bacteria (1.92×10^6 CFU/ml) mixture was transferred into dishes containing RAW 264.7 cells (6×10^5) and incubated (37°C, 5% CO₂, 30 min). Each culture dish was washed and treated with dimethyl sulfoxide (DMSO, 0.1%), rifampin (5 mg/L), ML141 (10 µM), and rifampin and ML141 co-treatment (5 mg/L, 10 µM), and incubated (37°C, 5% CO₂, 1hr). Following the treatment, plates were washed and treated with gentamicin (500 µg/ml,) and incubated (37°C, 5% CO₂, 30 min). Next, plates were washed 3 times. RAW 264.7 cells were lysed by incubation in chilled sterile water (5 min,). After incubation, each dish was washed forcefully with solution in the dish, and plated onto blood agar plates (37°C, 5% CO₂, 24 hr). The following day, colony counts were used to quantify colony forming units (CFU)/ml.

Statistical Analyses. Data from the invasion assay were collected and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison, where $P < 0.05$ is considered significant.

Results

Rifampin and ML141 co-treatment decreases *S. pyogenes* intracellular infection more than ML141 alone. However, there was no significant difference in rifampin alone compared to rifampin and ML141. Therefore, we hypothesize decreasing the concentration of rifampin will result in rifampin and ML141 having a significant difference in comparison to rifampin alone.

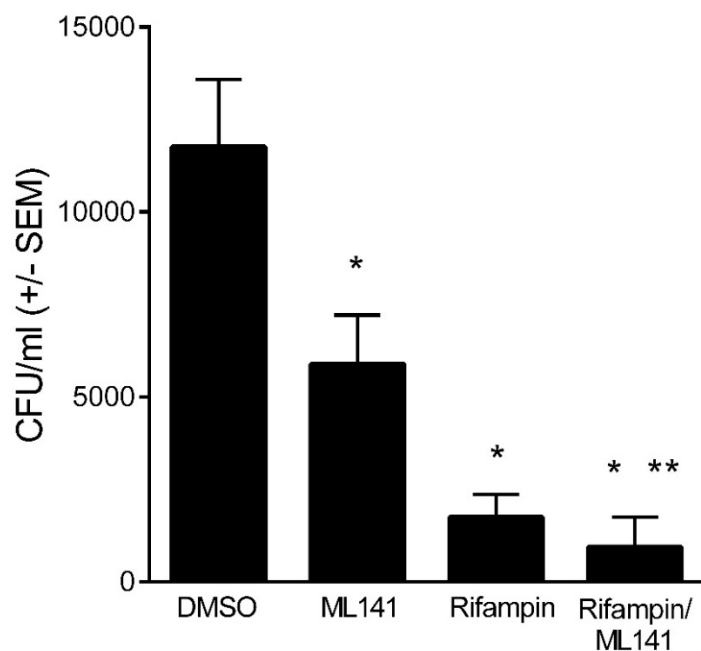


Figure. 1. Rifampin and co-treatment ML141 decreases *S. pyogenes* intracellular bacterial infection. *S. pyogenes* were cultured (200 rpm, 37°C, 24 hr) in Todd Hewitt Broth and subcultured before doing the assay. To begin the assay, bacteria were pelleted (10000 rpm, 37°C, 3 min) and washed. The appropriate concentration of bacteria (1.92×10^6 CFU/ml) was

transferred into dishes containing RAW 264.7 cells (6×10^5) and incubated (37°C, 5% CO₂, 30 min). RAW cells were treated with dimethyl sulfoxide (DMSO), rifampin (5 mg/L), ML141 (10 µM), and rifampin and ML141 co-treatment (5 mg/L, 10 µM) and incubated (37°C, 5% CO₂, 1hr). Following treatment, plates were washed and treated with gentamicin (500 µg/ml), and incubated (37°C, 5% CO₂, 30 min). Following extensive washing, RAW 264.7 cells were lysed by incubation in chilled sterile water (5 min). After incubation, each dish was washed forcefully with solution in the dish, and serial dilutions were plated onto Sheep Blood Agar plates (37°C, 5% CO₂, 24 hr). Colony forming units (CFU)/ml were quantified. One-way ANOVA followed by Tukey's multiple comparison was performed to test for significance, where $P < 0.05$ is significant (*less than DMSO control, ** less than ML141 alone).

Discussion:

This study consisted of an investigation on the effects of rifampin and ML141 co-treatment on *S. pyogenes* infections in RAW cells. Results indicated that rifampin and ML141 co-treatment decreases *S. pyogenes* intracellular infection more than ML141 alone (figure 1). However, there was no significant difference in rifampin alone compared to rifampin and ML141 (figure 1). ML141 decreases intracellular infection by *Streptococcus pyogenes* (unpublished). Rifampin also decreases intracellular infection by *S. pyogenes* (figure1). The problem is that rifampin and ML141 alone do not get rid of all the infection caused by *S. pyogenes* in the cell, and both are toxic to cells when used in high concentrations [1, 2]. As indicated in figure 1, rifampin and ML141 co-treatment had a greater decrease in *S. pyogenes* bacterial colony counts compared to ML141 alone and rifampin alone. This suggests *S. pyogenes* recurrent infections to be less likely to occur in patients treated with rifampin and ML141 co-treatment. Further research using different concentrations of rifampin with ML141 co-treatment will have to be investigated to

determine if a lower dosage of rifampin would be effective in decreasing *S.pyogenes* intracellular bacteria. Furthermore, rifampin and ML141 co-treatment inhibiting *S. pyogenes* intracellular infections will lead to a better understanding of *S. pyogenes* recurrent infections.

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